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Inhibitory Effect of Tannic Acid on Iron–Dextran-Augmented and 7,12-Dimethyl Benz(A)Anthracene-Initiated Skin Carcinogenesis

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Tannic acid (TA) is naturally occurring polyphenol present in fruits and vegetables. In this study we report that TA inhibits the carcinogenic effect of 7,12-dimethylbenz(a)anthracene (DMBA) in normal and iron-overloaded mice skin. Albino Swiss mice were given iron–dextran and pretreated with a single topical application of tannic acid; after 1 h, tumors were initiated by multiple topically application of DMBA. Appearance, number, and percent tumor incidence were recorded. When compared with the control group, the pretreated groups showed a significantly higher inhibition of tumor incidence. The induction of [3H]thymidine incorporation in cutaneous DNA and lipid peroxidation was inhibited in TA-pertreated animals as compared with the normal control group. Based on this study, we propose that TA significantly inhibits the augmentation potential of iron–dextran. A depletion in lipid peroxidation levels in TA-pretreated groups indicates that excessive generated oxidants in the mice skin tissues may be quenched by TA and because of the chelation of redox active iron and its faster elimination from the body. In conclusion, our data suggest that TA may be an effective chemopreventive agent and may offer protection against iron mediated skin cancer. J. Trace Elem. Exp. Med. 17:21–29, 2004.

Key words: tannic acid; iron overload; 7,12-dimethylbenz(a)anthracene; skin cancer

INTRODUCTION

There is increasing interest in identification of naturally occurring substances that may be capable in diminishing the tumorigenicity of the environmental car-

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cinogens. Tannic acid (TA) is naturally occurring plant phenol present in fruits and vegetables [1]. In addition to its use as an additive in medicinal products for humans, it has been used for the treatment of burns, diarrhea, and chemical antidotes in poisoning and as a local astringent [2]. It has been shown to be antioxidant and is a potent antagonist of the mutagenicity of polycyclic aromatic hydrocarbons (PAHs) such as benzo(a)pyrene (BP) [3,4]. According to the extensive experimental data it is now known that PAHs must be metabolically activated by peroxy radical dependent pathway and the electrophilic bay-region diol-epoxides act as the ultimate carcinogenic metabolites of PAH [5]. Consistent with this hypothesis during the past several years, naturally occurring plant phenols, including TA, have shown to inhibit the mutagenicity of (+)-7β, 8α-dihydroxy-9, 10-α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE-2), an ultimate mutagenic and carcinogenic metabolite of BP. It has been shown that TA possesses exceptionally high antimutagenic activity as compared with hydroxylated anthraquinones and cinnamic derivatives against BPDE-2 mutagenesis [4,6]. In addition, the pretreatment of animals with TA inhibits 7,12-dimethylbenz(a)anthracene (DMBA), benzo(a)pyrene, 3-methyl cholanthrene, and N-methyl-N-nitrosourea-induced tumorigenesis in mice. Aside from its effect on cytochrome P-450 dependent mono-oxygenase activity, it also potentates quenching free radicals and inhibits lipoxygenase activity induced by tumor promoters [7,8]. In a subsequent study, it has been reported that TA as well as other polyphenols, including quercetin and green tea polyphenols, inhibit skin tumorigenesis [9]. When it is applied topically, injected, or added to the diet or drinking water can decrease the risk of tumorigenesity in the skin and other organs [10]. Based on these studies, it appears that TA acts on different sites by different mechanisms and inhibits the manifestation of carcinogenesis. Previously we have was reported that iron acts as a mild tumor promoter in skin in addition to augmenting the effect of benzyl peroxide-mediated cutaneous tumorigenesis [11,12], and in another study we showed that low iron levels in the skin and during pregnancy are associated with reduced tumor incidence [13]. It is quite possible that antioxidant potential of TA may quench the excessively generated oxidant and inhibit tumor incidence. In this study, we provide the first evidence showing the inhibitory effect of TA on DMBA-induced skin tumorigenesis in normal and iron overloaded animals.

MATERIALS AND METHODS

Chemicals

Iron–dextran was purchased from the Red Cross (Iran) and hair-removing cream was from Tolidaro, Iran. DMBA and [3H]thymidine (specific activity 82 Ci/mmol) was purchased from Amersham Corporation, UK. All other chemicals and biochemicals used in this study were either of analytical grade or of highest purity grade available commercially.

Animals

Swiss albino female mice were procured from Central Animal House Facility of Tabriz University of Medical Sciences and used throughout this study. Ani-

mals were housed in an air-conditioned room in polypropylene cages and for long term chronic experiments, usually 20 mice and for acute experiment, six mice were housed in each group. They had a free access to pellet diet and water ad libitum. The animals were kept at room temperature of 24°C (± 2°C) and were exposed to alternate cycles of 12 h of light and 12 h of darkness. The dorsal skin of the mice was shaved with an electric clippers followed by the application of hair removing cream, at least two days prior to the treatment. Only mice that did not show signs of hair regrowth were used.

Treatment of Animals and Tumorigenesis Studies

For the tumorigenesis studies, 80 female swiss mice were divided into four groups. Groups I and II received normal saline intramuscularly (0.1 mL per mouse in hind limb) for a period of 2 weeks and served as controls. Groups III and IV animals received iron–dextran injections intramuscularly for a period of 2 weeks (1 mg iron/0.1 mL saline/mice/day). Twenty-four hours after the last injection of saline or iron–dextran, group II and IV animals were treated with TA (400 μg/200 μL acetone). One hour after the treatment of TA, the animals of all the groups received topical applications of DMBA (40 μg/200 μL acetone/mouse/day, for 5 days) on the dorsal skin. No further treatment was given. The mice were observed weekly for the incidence of tumors, which were recorded and plotted as function of weeks on test. The data were presented both as number of tumors per mouse as well as percent incidence of tumorigenesis.

For biochemical studies, animals were divided into four groups having six animals in each group. Group I and III animals received an intramuscular injection of saline. Groups II and IV were given a single injection of imferon (5 mg iron/mouse, given in two half doses on left and right hind limbs). Twenty-four hours after the saline or iron treatment, group I and II animals received a topical application of acetone (200 μL/mouse) whereas, group III and IV animals received a single topical application of TA (400 μg/200 μL acetone/mouse). One hour after the treatment of acetone or TA, all the animals were killed and their skins were removed and processed for biochemical estimations.

Tissue Preparation for Biochemical Assays

Tissue preparation was performed by using the method of Mohandas et al. [14]. After the desired time period, control and treated animals were killed by cervical dislocation. The animal skin was immediately removed, washed in ice-cold saline (0.9% NaCl) and the extraneous material was removed. All subsequent operations were carried out on ice at 4°C. For biochemical studies, a known amount of tissue was minced and homogenized in a polytron homogenizer. For biochemical estimation, post mitochondrial supernatant were used.

Assay of Lipid Peroxidation

The assay for microsomal lipid peroxidation was performed using the method of Wright et al. [15]. The reaction mixture in a total volume of 1.0 mL contained

0.58 mL of phosphate buffer (0.1 M, pH 7.4), 0.2 mL of microsome obtained from the homogenate (10% w/v), 0.2 mL of ascorbic acid (100 mM), and 0.02 mL of ferric chloride (100 mM). The reaction mixture was incubated at 37°C in a shaking water bath for 1 h. The reaction was stopped by the addition of 1 mL of TCA (10%). After the addition of 1.0 mL of TBA (0.67%), all the tubes were placed in a boiling water bath for a period of 20 min. In the end, tubes were shifted to crushed ice bath and then centrifuged at 2500 × g for 10 min. The amount of TBA reacting species formed was assessed by measuring the optical density of the supernatant at 535 nm using spectrophotometer (Milton Roy-21D) against a reagent blank. The results were expressed as nmol MDA formed/hr/gm of tissue at 37°C by using molar extinction coefficient of $1.56 \times 105\ M^{-1} \cdot cm^{-1}$.

[3H]Thymidine Incorporation Assay

For [3H]thymidine incorporation studies, the isolation of skin DNA and incorporation of [3H]thymidine DNA were performed using the method of Smart et al. [16]. Briefly, the skin was quickly removed, cleaned free of extraneous material including dermis, and 10% homogenate (w/v) was prepared in ice-cold water containing an equal volume of ice-cold TCA (10%). The precipitate thus obtained was washed with cold TCA (5%) and incubated with cold PCA (10%) at 4°C overnight. After incubation, it was centrifuged and the precipitate was washed with cold PCA (5%). The precipitate was dissolved in warm PCA (10%) followed by incubation in a boiling water bath for 30 min. It was then filtered through a Whatman 50 filter paper. Upon addition of scintilation fluid, the filtrate was used for [3H]thymidine counting in liquid scintillation counter (LKB-Wallace-1410). The amount of DNA in the filtrate was estimated by the diphenylamine method of Giles and Myers [17].

Statistical Analysis

The level of significance between different groups are based on Dunnett's *t* test followed by Analysis of variance test if necessary. The level of significance was chosen at $P < 0.05$.

RESULTS

The pretreatment effect of TA on iron dextran induced lipid peroxidation in normal and iron overload mice is given in Figure 1. Iron overload has been shown to augment lipid peroxidation in comparison with saline control and TA-treated groups of animals. However, the previous topical application of TA diminished the induction in lipid peroxidation significantly ($P < 0.05$). The effect of TA in [3H]thymidine incorporation into epidermal DNA is shown in Figure 2. A twofold increase in [3H]thymidine incorporation in mice receiving iron was observed, whereas this effect was significantly decreased by TA treatment.

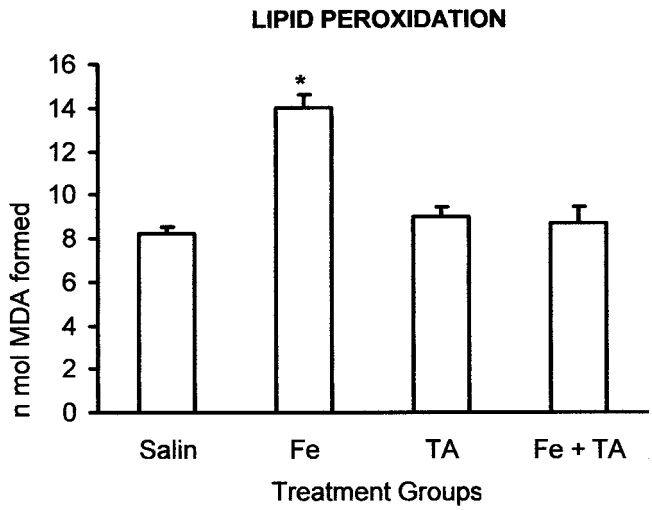

Fig. 1. Effect of TA on cutaneous lipid peroxidation. The animals were given 400 μg of TA and 5 mg of iron and were killed after 24 h of treatments. The exact treatment protocol and other experimental details are given in the text. Each value represents mean ± SE of six animals. $*P < 0.05$ when compared with saline-treated control mice.

The effect of pretreatment of TA on the tumor yield and tumor incidence is shown Figures 3 and 4, respectively. Figure 3, indicates that the tumor yield is decreased significantly in normal and iron overloaded animals by pretreatment with TA. By week 22, the tumor yield in the normal and iron overloaded animals was 1/mouse and 0.2/mouse, respectively. The maximum number of tumors in the iron-overloaded group by week 35 was 1.5/mouse, and in TA-treated animals, there was more than a threefold reduction, whereas in those groups of animals who received TA before the DMBA treatment there was no tumor incidence. However, in corresponding groups of animals receiving only acetone and iron alone, the tumor incidence was zero. In fact, these groups of animals did not develop any tumors during their life span (35 weeks' observation). Similarly, the effect of TA on percentage tumor incidence in normal and iron-overloaded animals is shown in Figure 4. By week 22, iron overload enhanced the tumor incidence up to 55%, whereas in at this time the incidence was about 25% in those animals receiving DMBA alone, and in the corresponding groups when the animals were pretreated with TA the incidence was significantly inhibited and there was no observation of tumors. At the end of week 35, the observed incidences in iron-overloaded, DMBA alone, and in iron-overload TA-pretreated animals the noted observations were as 70%, 40%, and 10% respectively. However, during this time also there was no tumor incidence among the DMBA-initiated and TA-pretreated group.

DISCUSSION

Cancer prevention is becoming popular because of the limited success in the treatment of cancer. Recently, dietary manipulations that affect the development

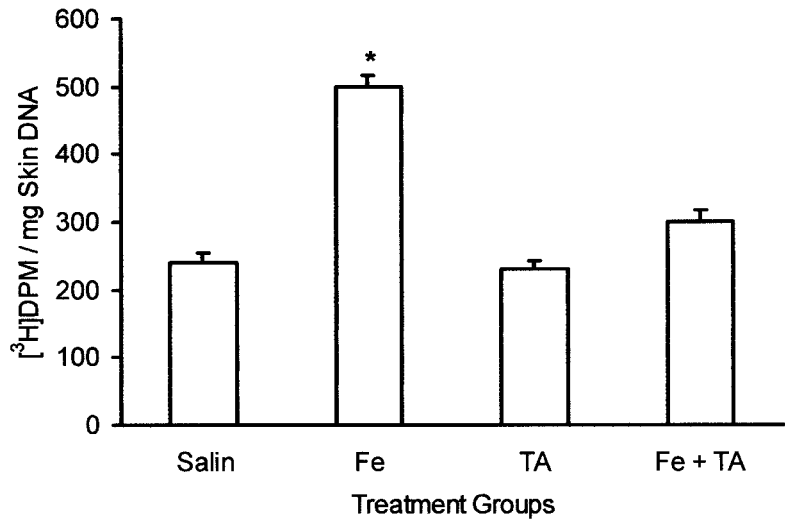

Fig. 2. Effect of TA on the inhibition of [3H]thymidine incorporation in cutaneous DNA in mice. The animals were given 400 μg of TA and 5 mg of iron and were killed after 24 h of treatments. The exact treatment protocol and other experimental details are given in the text. Each value represents mean ± SE of six animals. $*P < 0.05$ when compared with saline-treated control mice.

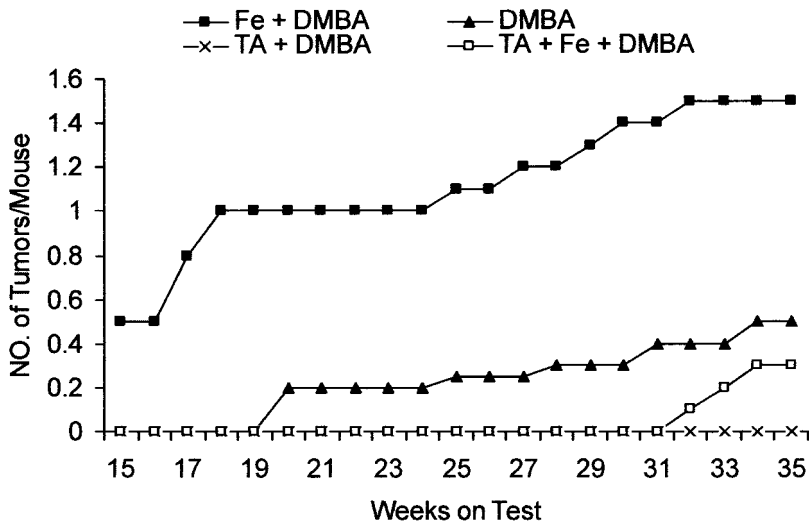

Fig. 3. Effect of TA on yield of tumors that developed as a result of iron overload and DMBA treatment in mice. The mice were given daily injections of either saline or 1 mg of iron–dextran for 15 days as described under the Materials and Methods section. Exact treatment protocol and other experimental details are given in the text. The tumors were counted every week and represented as number of tumors/mouse plotted as a function of weeks on test. Each value represents mean of number of tumors/mouse calculated on the basis of 20 animals.

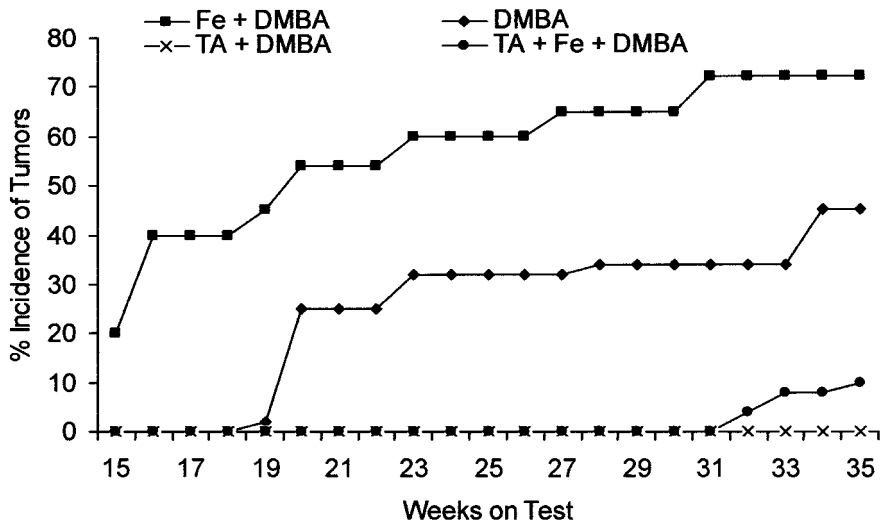

Fig. 4. Effect of TA on percent incidence of tumors as a result of DMBA-treated and iron overload mice. The data were recorded every week and plotted as a function of weeks on test. The mice were given daily injections of either saline or 1 mg of iron–dextran for 15 days as described under the Materials and Methods section. Exact treatment protocol and other experimental details are given in the text. Each value represents % incidence data calculated on the basis of 20 animals.

of cancer have been highlighted. A large number of studies have been conducted to ensure that diet plays an important role in the etiology and development of cancer. Several epidemiological studies have also suggested that diet influences human cancer risk [18]. A large number of chemical compounds having anti-carcinogenic potential have been identified and shown to be present in normal human diet [19]. These compounds are predominantly plant products, which have highly diversified chemical structures. Polyhydroxy plant phenols, such as TA, are one classic example of such agents that are present in a variety of plants [20]. Many of these are consumed in human diet [21]. TA has also been shown to possess beneficial pharmaceutical effects [22,23]. It has been shown that dietary supplementation of TA results in the protection against the onset of forestomach, lung, and skin neoplasia, but the effect was more pronounced in forestomach followed by lung and skin [24]. Recently it has been shown that TA inhibits the skin tumor promotion effect of Ultraviolet B radiation [25]. It has also been reported that TA is involved in direct binding to DNA, which also plays an important role in the initiation of tumor formation by poly cychlic aromatic hydrocarbones [26].

The mechanism of protection in these studies seems to be caused by the inhibition of PAH-metabolizing enzymes and their subsequent binding to DNA. The observed decrease in the tumor yield and tumor incidence in animals receiving a pretreatment of TA before the application of DMBA also suggests that the inhibition in skin tumorigenesis in iron-overloaded animals may be because of the inhibition in the metabolism of DMBA at the initiation stage. Such an inhibition may lead to the diminution of a number of initiated cells that might

ultimately clonally expand to give large number of tumors. Therefore, the removal of peroxy radical generated in iron overload animals, which may be involved in the higher production of activated metabolite of DMBA, may be responsible for the decrease in tumorigenesis response. The ability of TA to inhibit tumor incidence is evident from the data of the present study, where the previous application of TA before the application of DMBA in normal and iron-overload animals lowering of the enhanced lipid peroxidation. In this study TA is shown a potent inhibitor of papilloma formation in mouse skin. Thus, the observed decrease may be because of the abrogation of oxidative stress by TA induced in benign papilloma by iron overload in the present study. However, the interaction of TA with iron in iron-overloaded animals and its subseqent chelation with the iron, thus rendering it ineffective in participating free radical generation, cannot be ruled out. Therefore, the chelated iron with TA may be eliminated fast from the body as compared with unchelated iron, which remains for a longer duration of time in tissues. Furthermore, the diminishing level of lipid peroxidation and [3H]thymidine incorporation in cutaneous DNA in TA pretreated animals indicates that the direct binding involvement of TA to DNA also play an important role in the initiation of tumor formation by DMBA.

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